Ecotoxicology and Environmental Safety 73 (2010) 1842–1851

Contents lists available at ScienceDirect

Ecotoxicology and Environmental Safety

journal homepage: www.elsevier.com/locate/ecoenv

Biochemical effects and polycyclic aromatic hydrocarbons (PAHs) in senegal sole (*Solea senegalensis*) from a Huelva estuary (SW Spain)

M. Oliva [a,*], M.L. González de Canales [a], C. Gravato [b,c], L. Guilhermino [b,c], J.A. Perales [d]

[a] Department of Biology, Marine and Environmental Science Faculty, University of Cádiz, Avda. República Saharaui S/N, Puerto Real 11510, Cádiz, Spain
[b] University of Porto, CIMAR-LA/CIIMAR & ICBAS, Centro Interdisciplinar de Investigação Marinha e Ambiental, Laboratório de Ecotoxicologia, Rua dos Bragas 289, 4050-123 Porto, Portugal
[c] ICBAS, Instituto de Ciências Biomédicas de Abel Salazar, Laboratório de Ecotoxicologia, Lg. Prof. Abel Salazar 2, 4099-003 Porto, Portugal
[d] Department of Environmental Technologies, CACYTMAR, Andalusian Centre of Marine Sciences and Technology, University of Cadiz, 11510 Puerto Real, Cadiz, Spain

ARTICLE INFO

Article history:
Received 2 June 2010
Received in revised form
24 August 2010
Accepted 25 August 2010
Available online 16 September 2010

Keywords:
Stress oxidative
Biomarkers
PAH's
Sole
Estuary

ABSTRACT

Relations between several stress oxidative biomarkers and aromatic polycyclic hydrocarbon (PAH) concentrations have been studied in wild sole, *Solea senegalensis* collected in the vicinity of a petrochemical industry. Antioxidant enzyme activities in eco-toxicological studies constitute excellent markers for exposure to a large variety of pollutants. The 16 PAHs in sediment as well as oxidative damage (LPO), activity of catalase (CAT), glutathione peroxidase (GP*x*), glutathione S-transferase (GST), glutathione reductase (GR) and PAHs type metabolites in sole liver were analysed. Significant correlations ($p < 0.05$) were established between some biomarkers as GST, GP*x* and CAT and PAHs metabolites in liver (naphthalene, pyrene and phenanthrene) and PAHs concentrations in sediments (fluoranthene, acenaphthene, anthracene and chrysene). PAHs accumulated in the sediment and organisms are inducers of antioxidant defences. GST, GP*x* and CAT were robust biomarkers showing correlations with both PAHs in sediments and liver PAH metabolites showing different responses to low and high molecular weight PAHs.

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are the most widespread organic pollutants. In addition to their presence in fossil fuels, they are also formed by an incomplete combustion of carbon-containing fuels such as wood, coal, diesel, fat, tobacco, or incense. PAHs are found wherever there is oil pollution and combustion wastes. Sediments of many marine and freshwater harbours and even remote ocean locations are contaminated with PAHs.

PAHs and their halogenated forms are chemically stable, and due to their lipophilic nature they can easily penetrate biological membranes and accumulate in organisms. PAHs are important environmental pollutants because of their ubiquitous presence and carcinogenicity. The United States Environmental Protection Agency (USEPA) and World Health Organization (WHO) have identified 16 PAHs as priority pollutants, some of these, e.g. benzo(a)anthracene, chrysene and benzo(a)pyrene are considered to be potential human carcinogens (Tuvikene, 1995).

PAHs reveal their toxicity following biotransformation to toxic metabolites (Varanasi and Stein, 1991; Stein et al., 1992) through metabolic activation (one- or two-electron oxidation) in the organism (Cavalieri and Rogan, 1985).

In aquatic systems, PAHs tend towards an increased toxicity with increased molecular weight (Eisler, 1987). In addition, although the rate of uptake from the environment is variable among species, bioaccumulation tends to be rapid. The degree of ecosystem contamination by toxic organic chemicals can be estimated by the analysis of biochemical changes.

Biomarker can be defined as 'the measurements of body fluids, cells, or tissues that indicate in biochemical or cellular terms the presence of contaminants or the magnitude of the host response'. A more generalized version which would also accommodate whole animal studies would include 'measurements on whole animals' and 'indicate in physiological, behavioural or energetic terms' (Sarkar et al., 2006).

The biomarkers (biochemical and physiological) that reflect the health status of fish at lower organizational levels (molecular and cellular), respond effectively to chemical stress and have high toxicological relevance, while those that reflect health conditions at higher organizational levels (e.g. condition indexes) respond slowly to stress and have lower toxicological relevance (Adams et al., 1989).

* Corresponding author. Fax: +34 956 016 019.
E-mail address: milagrosa.oliva@uca.es (M. Oliva).

0147-6513/$ - see front matter © 2010 Elsevier Inc. All rights reserved.
doi:10.1016/j.ecoenv.2010.08.035

Oxidative stress is one of the primary cellular mechanisms resulting from an exposure to PAHs and the subsequent increase in prooxidative processes (Machala et al., 2001). Peroxidation of membrane lipids and oxidative damage to other key cellular components can lead to genotoxicity, promotion of carcinogenesis, and perturbation of several other signal and metabolic pathways (Klaunig et al., 1998).

The first step in the xenobiotics metabolism is usually catalyzed by cytochrome P450-dependent monooxygenases (phase I) and their products are subsequently coupled to endogenous metabolites (phase II) (Landis and Yu, 1995). Exposure to PAHs results in the induction of specific forms of cytochrome P4501A that catalyzes aryl-hydrocarbon-hydroxylase (AHH), ethosyresorufin-O-deethylase (EROD) and 7-ethopxy-coumarin-O-deethylase (ECOD) activity (Di Giulio et al., 1993). PAHs are subject to biotransformation in a first step by enzymes of the P450 system. An induction of cytochrome P4501A (CYP1A) has been found in several species exposed to PAHs, including *Leuciscus cephalus* (Machala et al., 2001), *Dicentrarchus Labrax* (Gravato and Santos, 2003), *Gadus morhua* (Sturve et al., 2006), *Pomatoschistus microps* (Vieira et al., 2008), *Symphodus melops* (Almroth et al., 2008) and *Carassius auratus* (Lu et al., 2009).

PAHs through non-redox cycling have the potential to produce reactive oxygen species (ROS) that overcome the protection afforded by antioxidant defence mechanisms, thereby leading to an oxidative damage, which is manifested by damage to tissue macromolecules including DNA, proteins and lipids (Ahmad et al., 2004; Shi et al., 2005). ROS production is usually deduced through the changes of antioxidant systems.

Antioxidant enzymes (Phase II) have been proposed as biomarkers of contaminant-mediated oxidative stress in a variety of marine organism, and their induction reflects a specific response to pollutants (Cossu et al., 1997). Earlier usefulness of glutathione-dependent enzymes and other antioxidant enzymes as markers for oxidative stress, produced by PAHs in fish, has been reported by many workers (Jifa et al., 2006; Almroth et al., 2008; Silva et al., 2009).

On the other hand, results obtained by Reynaud et al. (2002) in *Cyprinus carpio* suggest that the induction of macrophage oxidative function may be an equally sensitive marker of PAHs exposure as the induction of biotransformation activities and confirm that responses mediated by the Ah-receptor are similar, if not identical, to those of mammals. A relation between animal biotransformation and immune system has also been observed. There are numerous functional interrelationships between these two systems (Reynaud et al., 2008). In fish, Carlson et al. (2004) have demonstrated that immune cells have all the machinery responsible for PAH metabolism.

Field experiments are usually performed to determine whether metabolic enzymes can be used as biomarkers of effects after months or years, following an oil spill event (Gagnon and Holdway, 1999). Antioxidant enzymes thus play a crucial role in maintaining cell homeostasis. These enzymes have been proposed as biomarkers of contaminant-mediated oxidative stress in a variety of marine organisms, and their induction and/or inhibition reflect/s a specific response to pollutants (Jee and Kang, 2005).

Some adult benthic fish, such as sole, come into direct contact with sediments, and eat benthic invertebrates that accumulate PAHs.

Solea senegalensis is a benthonic marine species living in sandy or muddy bottoms, off coastal areas up to 100 m depth, in brackish lakes and estuaries. Senegal sole feeds basically on benthonic invertebrate, such as larvae from polychaets, bivalve molluscs and small crustaceans. Sexual maturity is reached when the size is 30 cm. Spawning happens between the months of March until June (Froese and Pauly, 2010). *S. senegalensis* is a well adapted species to warm climates and is commonly exploited in an extensive aquaculture production in Spain and Portugal (Drake et al., 1984; Dinis, 1992) and have been used in field and laboratory toxicity assays being a sensitive specie to pollutants (Jimenez-Tenorio et al., 2008; Oliva et al., 2009; Costa et al., 2009).

As far as we know, few studies have evaluated the effect of PAHs on stress oxidative enzymes in *S. senegalensis* (Jimenez-Tenorio et al., 2008; Solé et al., 2008).

In the present work, relations between PAHs and stress oxidative biomarkers have been studied in *S. senegalensis* from the Huelva estuary. The work describes an in situ toxicity assessment approach for improved characterizations of PAHs exposure and effects along time. Stress oxidative enzymes were used in order to assess sediment toxicity of an estuary, chronically affected by PAH spills.

2. Material and methods

2.1. Sampling area

The Ría of Huelva is located on the southern Spanish Atlantic coast, where the Odiel (H1) and Tinto (H2) Rivers join to form the Padre Santo Canal (H3), which drains into the gulf of Cadiz forming a wide estuary (Fig. 1). The estuary receives contaminating inputs from a mining and industrial area in the estuary where an important petroleum refinery is located (Jiménez-Tenorio et al., 2007; Vicente-Martorell et al., 2008). Four samplings were conducted from October 2004 to May 2006: two of them were realized at autumn (October 2004 and October 2005) and the others at spring (April 2005 and May 2006). During each sampling sediment and fish samples of the three different sampling sites were obtained.

2.2. Organisms

Specimens of Senegal sole *S. senegalensis* were transported in aerated tanks to Mazagon's port (Huelva) (less than 1 km) and dissected immediately. A total of 97 (125.04 ± 27.12 g weight, 23.14 ± 1.8 cm length) fish were dissected and samples of liver of each fish were taken: 43 samples representing Odiel river (24 samples for the autumn and 19 samples for the spring season), 34 samples representing Tinto river (16 samples for the autumn and 18 samples for the spring season) and 20 samples representing Padre Santo Canal for autumn season (in the spring season no sole was captured in this site).

Ria of Huelva Estuary is a zone where fishing is prohibited due to aquatic pollution from what the Regional Government gave us a three days limited fishing special permission for every season. A trammel net anchored night before the sampling was used. Each night the net was located in one of the three different sampling sites studied. Trammel net is not a selective net, for this reason the number of sole specimens was variable in the different sampling sites. The number of specimens from selected species is a natural limitation when the objective is to study wild species.

Fish tissues samples were transported to the laboratory into nitrogen liquid and stored at $-80\,^{\circ}C$. Twelve specimens of *S. senegalensis* (182.52 ± 23.89 g weight, $25{,}27 \pm 0.78$ cm length), used like unpolluted or control fish, were obtained from the aquaculture facilities of the Faculty of Marine and Environmental Sciences (University of Cadiz, Spain). The experiments described comply with the Guidelines of the European Union Council (86/609/EU), the Spanish Government (RD 1201/2005), and of the University of Cadiz (Spain) for the use of animals in research.

2.3. Analytical procedure for PAHs

The 16 PAHs identified by United states Environmental Protection Agency (EPA) and World Health Organisation (WHO) as priority pollutants have been analysed in samples of sediment and hepatic tissue of *S. senegalensis*. The 16 PAHs (total PAHs–TPAHs-) have been divided in two different categories: low molecular weight PAHs–LPAHs- (naphthalene, acenaphthylene, acenaphthene, fluorene, phenantherene, anthracene, fluoranthene and pyrene) and high molecular weight PAHs–HPAHs-(benzo(a)anthracene, chrysene, benzo(b)fluoranthene, benzo(k)-fluoranthene, benzo(a)pyrene, dibenzo(a,h)anthracene, benzo(g,h,i)perylene and indeno(1,2,3-cd)pyrene).

PAH analysis in sediment was based on the analytical procedure proposed by the USEPA (1996, 2000). Approximately 2–4 g of sediment was weighed and treated with anhydrous Na_2SO_4. The samples were Soxhlet-extracted with 10 mL dichloromethane-acetone (8:2 v/v) for 24 h (6 cycles per hour). The extracts were purified on florisil columns, and the PAHs were eluted with 100 mL dichloromethane-hexane (2:8 v/v). The extract was concentrated to 1 mL using a rotary evaporator after changing the solvent from dichloromethane-hexane to

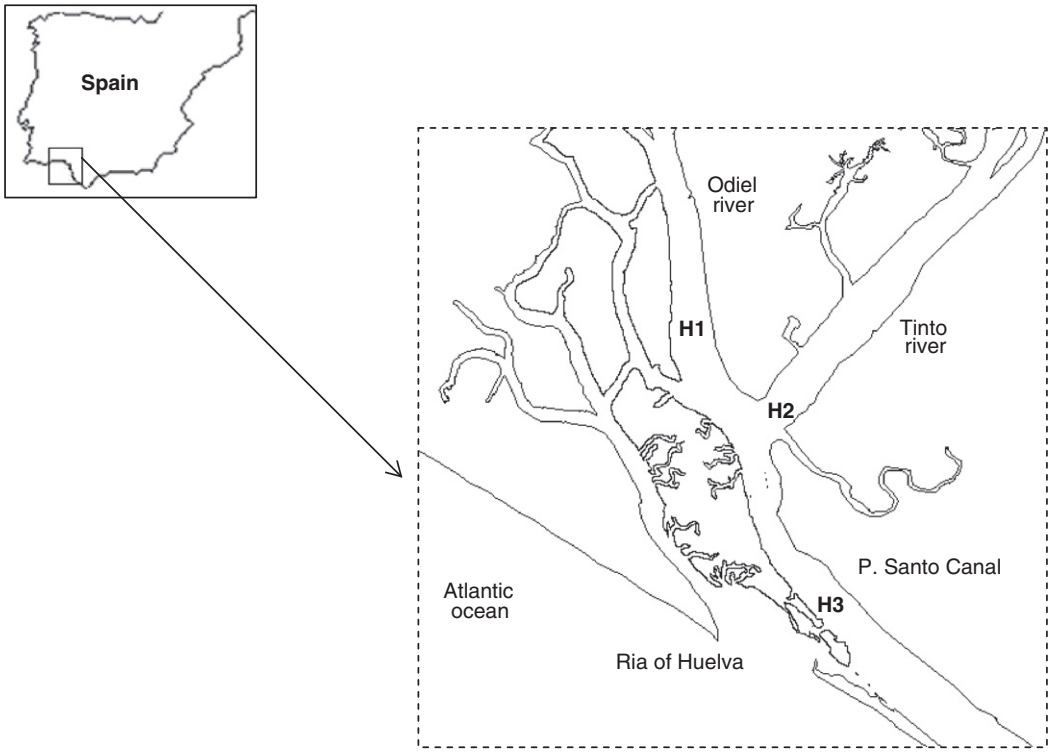

Fig. 1. Location map of sampling sites in Huelva estuary (SW Spain): H1 (Odiel river); H2 (Tinto river) and H3 (Padre Santo Canal).

acetonitrile. Na_2SO_4 was of an analytical grade and dichoromethane, hexane, acetone and acetonitrile were of HPLC grade.

The composition and concentration of 16 different PAHs were determined by HPLC. A standard solution, SUPELCO 47940-u PAH mix in acetonitrile (containing 16 individual PAHs), was used for quantification.

2.4. Fixed wavelength fluorescence measurements (FF)

Post-mitochondrial supernatant (PMS) of liver were further diluted in methanol 50% to 1:400 for an FF measurement. Fluorescent readings were performed for naphthalene-type metabolites at an excitation/emission 290/335 nm, benzo(a)-pyrene-type metabolites readings were made at 380/430 nm, for pyrene-type metabolites readings were made at 341/383 and for phenanthrene-type metabolites readings were made at 256/380. Liver cytosol metabolites are reported on the basis of milligram protein, as previously adapted by Gagnon and Holdway (2000).

2.5. Analysis of stress oxidative biomarkers

Liver was rapidly isolated, frozen and maintained at −80 °C until further analysis. Each liver was homogenized (1:10) in 0.1 M K-phosphate buffer (pH 7.4). Part of this liver homogenate was used to determine the extent of endogenous LPO by measuring the thiobarbituric acid reactive substances (TBARS), according to Ohkawa et al. (1979) and Bird and Draper (1984), with the adaptations of Filho et al. (2001) and Torres et al. (2002). The remaining liver homogenate was centrifuged for 20 min at 10,000*g* (4 °C) to obtain the post-mitochondrial supernatant (PMS). The GST activity was determined following the conjugation of GSH with 1-chloro-2,4-dinitrobenzene (CDNB) at 340 nm (Habig et al., 1974). CAT activity was determined in PMS and represents the H_2O_2 consumption obtained at 240 nm in the presence of H_2O_2 (Claiborne, 1985). The GP*x* activity was determined in PMS by measuring the decrease in NADPH at 340 nm and using H_2O_2 as a substrate (Mohandas et al. 1984). The GR activity was assayed in PMS according to Cribb et al. (1989). The protein concentration of liver PMS was determined according to Bradford (1976).

2.6. Statistical analysis

Statistical analyses were performed using the statistical software package STATISTICA (data analysis software system) version 7 (2004, Statsoft, Inc. USA).

For each data set, the assumptions of an analysis of variance (ANOVA), normality and equal variance, were checked using Shapiro-Wilks and Levene tests, respectively. If the assumptions were not met, the necessary transformations were performed. If data set has normality, one-way ANOVA were conducted and where significant, a post-hoc mean comparison test (Tukey's test) was realized. If non-normal data set was found, the Kruskal–Wallis and Man Witney (one-tail) tests to find out significant differences were conducted.

To analyse the correlation between biomarkers and PAH's concentrations and metabolites, Pearson and Spearman correlations test were used. All statistical analyses were conducted at an alpha level of 0.05.

3. Results

3.1. Correlationships between PAHs concentrations in sediments and PAHs metabolites in liver of S. senegalensis.

PAHs-type metabolites in liver of *S. senegalensis* are showed in Fig. 5. B(a)pyrene-type metabolites are PAHs with five aromatic rings. PAHs with five aromatic rings analysed in sediments in the present work were dibenzo(a)anthracene, benzo(b)fluoranthene and benzo(k)fluoranthene. Pyrene-type metabolites (four aromatic rings) concentrations in liver were correlated with analogues (four aromatic rings) analysed in sediment: benzo(a)anthracene, chrysene and fluoranthene. In the case of phenanthrene-type metabolites (three aromatic rings), the PAHs analysed was acenaphthylene, anthracene, acenaphthene and fluorene. Napththalene-type metabolites (two aromatic rings) analysed in liver were correlated with naphthalene concentration in sediments. Correlations between PAHs metabolites in liver and their analogues in sediment were conducted and a significant correlation was observed between phenanthrene-type metabolites in liver and fluorene concentration in sediment ($p=0.028$; $r=0.687$).

3.2. Stress oxidative biomarkers in liver

Significant differences ($p<0.05$) in the response of biomarkers between autumn and spring were found. The response was AUTUMN > SPRING to LPO, GR (Tinto) and GST and

SPRING > AUTUMN to GR (Odiel), CAT and GP*x*. On the other hand, significant differences ($p < 0.05$) between LPO, CAT and GR responses in control fish and fish from sampling sites were observed in Odiel and Tinto River. The concentrations of the different biomarkers in hepatic tissue are showed in Table 1.

3.3. Correlationships between PAHs concentrations in sediments and stress oxidative biomarkers.

PAHs concentrations in sediments of the different sampling sites are shown in Figs. 2–4. Significant correlations were established between several biomarkers and PAHs in sediments: GST and

Table 1
Stress oxidative biomarkers (average ± standard error) in different seasons (October: Autumn season; April and/or May: Spring season) and sampling sites. Units: LPO (nmol TBARS/g wt), CAT, GR, GST and GP*x* (nmol/min/mg prot). 1: Odiel River, 2: Tinto River, 3: Padre Santo Canal.

Date	Sampling station	*n*	LPO	CAT	GR	GST	GP*x*
October 2004	1	12	80.66 ± 23.52	16,783.87 ± 3056.49*	7.49 ± 0.56	5.67 ± 0.63	2.17 ± 0.32
	2	4	123.69 ± 25.29	12,537.53 ± 12,537.53*	8.30 ± 0.00	3.42 ± 0.50	1.87 ± 1.87
	3	8	70.15 ± 19.09	29,276,56 ± 103.91	8.19 ± 1.03	6.84 ± 1.44	3.05 ± 0.07
April 2005	1	9	62.28 ± 16.73	17,009.00 ± 2220.01*	9.50 ± 1.60	4.10 ± 0.36	1.53 ± 0.36
	2	6	12.06 ± 8.49	16,809.71 ± 2308.67*	7.90 ± 7.90	5.13 ± 0.25	2.19 ± 0.04
	3	0					
October 2005	1	12	339.01 ± 80.38*	17,884.73 ± 2229.73*	6.97 ± 0.67	6.00 ± 0.66	2.46 ± 0.39
	2	12	480.04 ± 50.37*	16,406.02 ± 1789.42*	10.59 ± 1.22	6.49 ± 0.29	2.11 ± 0.21
	3	12	147.70 ± 83.95	31,859.57 ± 8576.36	9.57 ± 1.43	8.55 ± 1.41	2.37 ± 0.27
May 2006	1	10	82.63 ± 8.35	30,804.37 ± 6620.63*	13.11 ± 2.13*	5.48 ± 0.63	3.69 ± 0.41
	2	12	116.40 ± 19.39	25,965.11 ± 2836.63*	7.14 ± 0.99	4.63 ± 0.47	3.115 ± 0.42
	3	0					
Control	Farm	12	57.79 ± 17.82	42,081.87 ± 5061.77	6.63 ± 0.87	5.25 ± 0.59	3.28 ± 0.63

* means are significantly different from the control ($p < 0.05$).

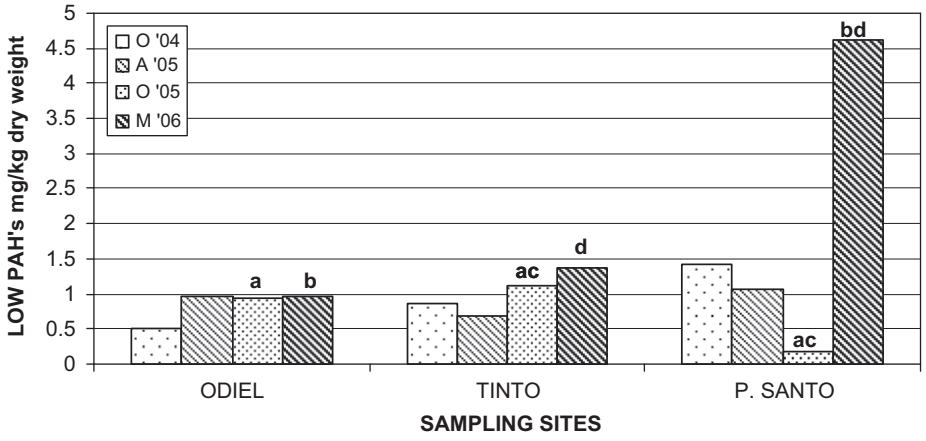

Fig. 2. LPAHs concentrations in sediments collected from the different sampling sites in 2004, 2005 and 2006 years. (O: October, A: April, M: May). Letters a, b, c, and d are used to show significant differences between PAH concentrations from the different sampling sites. Equal letter shows significant differences between sampling sites from the same season ($p < 0.05$).

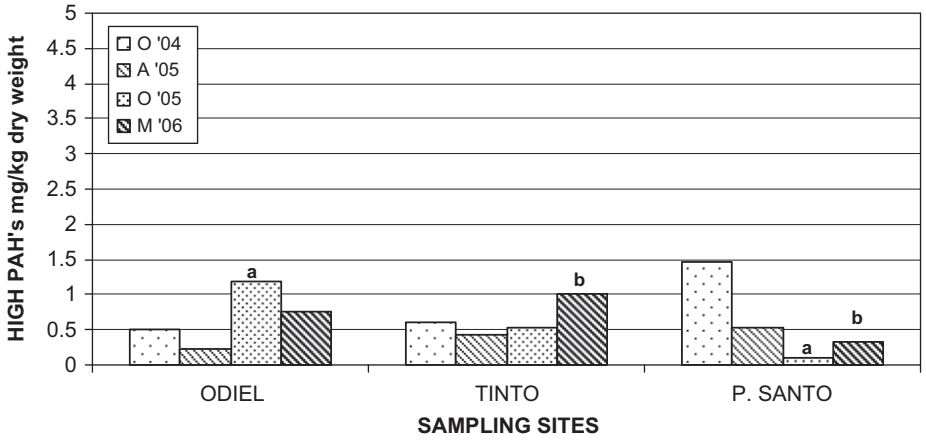

Fig. 3. HPAHs concentrations in sediments collected from the different sampling sites in 2004, 2005 and 2006 years. (O: October, A: April, M: May). Letters a and b are used to show significant differences between PAH concentrations from the different sampling sites. Equal letter show significant differences between sampling sites from the same season ($p < 0.05$).

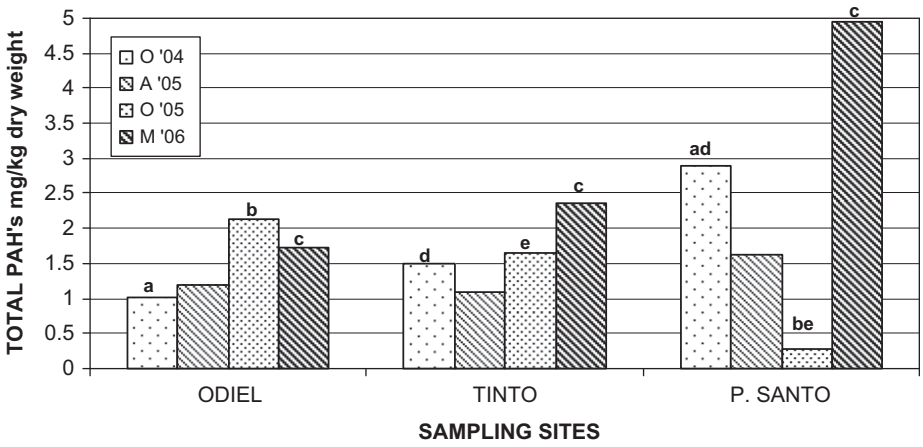

Fig. 4. TPAHs concentrations (low, high and summatory of 16 PAHs) in sediments collected from the different sampling sites in 2004, 2005 and 2006 years. (O: October, A: April, M: May). Letters a, b, c, d and e are used to show significant differences between PAH concentrations from the different sampling sites. Equal letter show significant differences between sampling sites from the same season ($p < 0.05$).

fluoranthene ($p=0.006$, $r=0.826$), chrysene ($p=0.005$, $r=0.804$), HPAHs ($p=0.022$, $r=0.709$) and TPAHs ($p=0.014$, $r=0.740$). On the other hand, GP*x* showed a significant correlation with acenaphthene ($p=0.010$, $r=0.765$) and CAT with anthracene ($p=0.027$, $r=0.692$).

3.4. Correlationships between PAHs metabolites in hepatic tissue and stress oxidative biomarkers

PAHs concentrations observed in hepatic tissue was very low, zero or undetectable in most samples (data unpublished), for this reason only correlations between PAHs metabolites in liver have been conducted (benzo(a)pyrene, pyrene, naphthalene and phenanthrene) and stress oxidative biomarkers.

PAHs metabolites in hepatic tissue of fish from different sampling sites are showed in Fig. 3. Significant correlations were established between GST and naphthalene-type metabolites ($p=0.045$, $r=-0.269$), and pyrene-type metabolites ($p=0.033$, $r=-0.0285$). GP*x* showed a significant correlation with phenanthrene-type metabolites ($p=0.023$, $r=-0.301$).

4. Discussion

On a global scale, quality criteria or quality normative in sediments have been developed principally in North America and have been used in countries such us United States, Canada, Australia, New Zealand or Honk Kong (Burton, 2002). In Europe, the principal reference is in the Holland legislation (Kamer, 1994; VROM, 2000). At present, the use of quality normative and the implementation of procedures of risk evaluation in marine sediments is scarce in the legislative frame of the European countries (Den Besten and Heise, 2007).

To establish the contamination grade for PAHs in the different sampling sites, the level of PAHs observed was compared with the Holand Environmental Quality Criteria to Marine Sediments (Kamer, 1994).

This criterion is established in basis to the summatory of 10 PAHs (naphthalene, phenanthrene, anthracene, fluoranthene, chrysene, benzo(a)anthracene, benzo(k)fluoranthene, benzo(b)-fluoranthene, benzo(g)perylene and indene). The summatory of those PAHs in Odiel River was 0.68, 0.82 mg/kg in the Tinto River and 1.81 mg/kg dry weight in Padre Santo Canal. Conform to this criteria, Odiel and Tinto River was classified as no or few contaminated sites and Padre Santo Canal is classified as moderately contaminated site, where a high concentration of LPAHs was observed in the sediment.

PAHs more abundant, in both sediment and liver, have been LPAHs. Significant correlation between phenanthrene-type metabolites and fluorene concentration in sediments was observed. This fact and the correlations observed between stress oxidative biomarkers and PAHs metabolites of low molecular weight (2 and/or three aromatic rings) in liver corroborate a higher accumulation of LPAHs in liver of *S. senegalensis*.

Several authors also observed a highest accumulation of LPAHs in fish and mollusc tissues (Narbonne et al., 1999; Salazar-Coria et al., 2007). The preferential accumulation of PAHs is determined by their solubility and bioavailability, related to the octanol–water partition coefficient (*K*08), molecular weight, exposure route and incorporation of the PAHs, which involves the habits of native biota (Conell and Miller, 1984). Schrap and Opperhuizen (1990) revealed that PAHs with high *K*08 values, such as pyrene and benzo[*a*]pyrene, bind to humic dissolved material or to other dissolved organic material present more than PAHs with low *K*08 values (anthracene or phenanthrene). The number of rings has been reported to influence the metabolic velocities of PAHs. Thus, the decrease was faster for PAHs of low molecular weight (anthracene and phenanthrene, which are rapidly released in the aqueous compartment) than for PAHs of high molecular weight (pyrene and benzo[*a*]pyrene), which are strongly adsorbed in sediment particles. When the rate of desorption from particles and humic material is rapid, uptake from interstitial water seems to prevail as for LPAHs; when the desorption rate is slow compared with the ingestion rate, then ingestion becomes more competitive as for HPAHs (Narbonne, 1999).

S. senegalensis is a flat fish with a practically sedentary life and a very territorial biology. This specie only realizes one migration per year to the coastal zone in the spawning period (March–June), when fish has achieved the reproductive size (25–30) cm. Juveniles, after completing the metamorphosis, are swept to the coast and go into the estuarine zones. The specimens captured in this work has a lower size than the reproductive size, for this reason, probably, sole specimens captured have remained around two-three years in the same zone. This prolonged contact with the estuary sediments justifies the study of the relation between PAHs concentrations in sediments and biomarkers in migratory specie, such as *S. senegalensis*.

Liver, but not bile, was used to measure PAHs metabolites due to the number of fish captured in each sampling site was limited (10–12 specimens). The gall bladder of *S. senegalensis* was very small and thin and the quantity of bile depends on the digestive

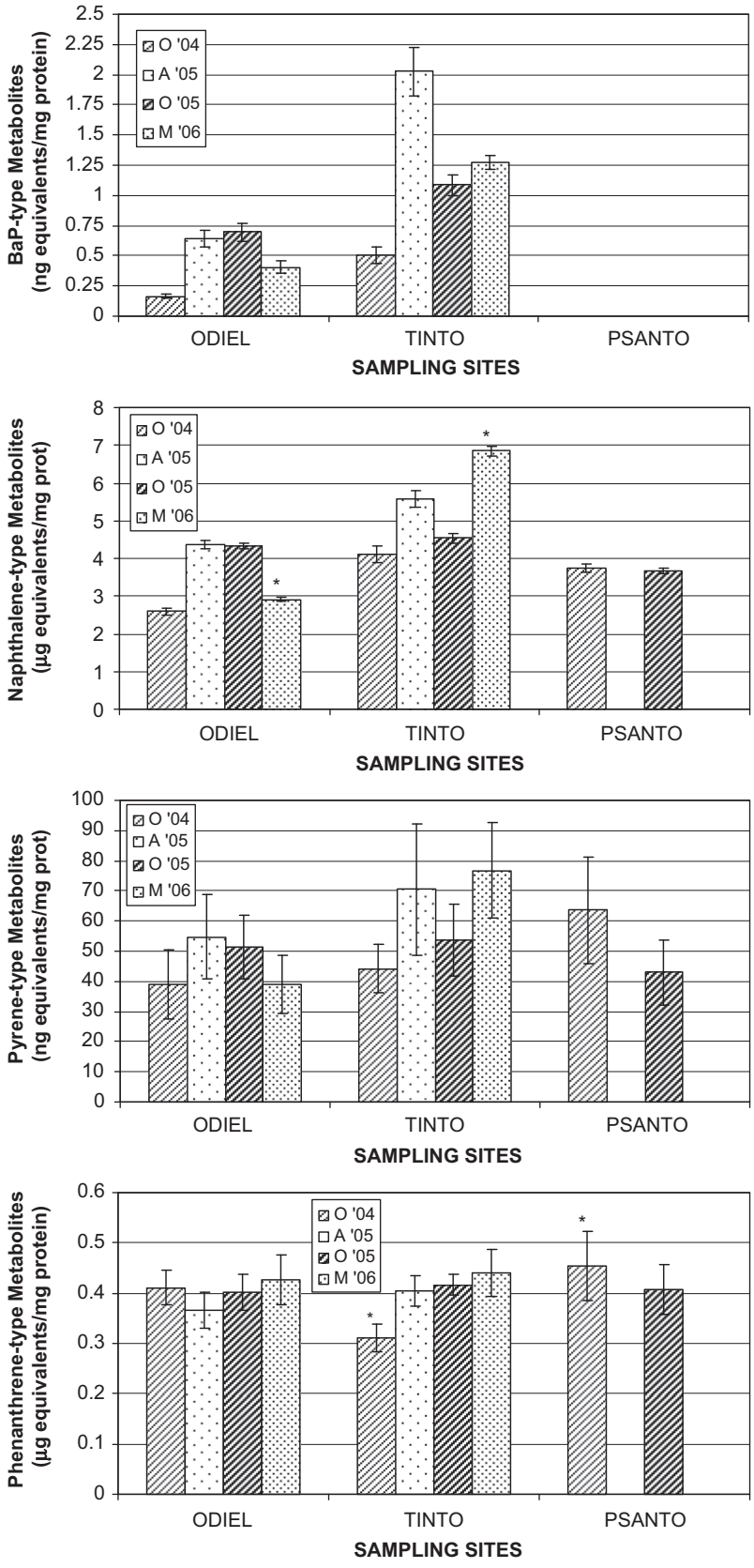

Fig. 5. PAHs metabolites (average and standard error) in liver of *S. senegalensis* captured from the different sampling sites in 2004, 2005 and 2006 years. (O: October, A: April, M: May). Asterisks denote means are significantly different in the same season ($p < 0.05$).

state of the fish. In this study, the quantity of bile was very low and usually insufficient to measure metabolites in some fish. It was necessary at least 4–5 gall bladders to realize one analysis. Authors preferred work with single organisms than pulls, because the number of pulls in each sampling site was insufficient to the statistical analysis.

PAHs are lipophilic compounds, but adipose tissue was scarce in the specimens studied, for this reason, PAHs metabolites was also measured in muscle in conjunction with other aerobic and anaerobic metabolism enzymes, but this information are being processed for a new paper.

The responses of stress oxidative biomarkers, in the present work, were more accused in the Odiel and Tinto River than the Padre Santo Canal. Being the Padre Santo Canal, the most PAHs contaminated site, an explanation for the high activity of biomarkers as LPO, CAT and GR in the Odiel and Tinto Rivers could be the presence of other pollutants, such as heavy metals (Vicente-Martorell et al., 2009). Heavy metals accumulation causes an increase in ROS leading to oxidative stress in fish (Roche and Bogé, 1993; Dautremepuits et al., 2002) promoting oxidative damage by directly increasing the cellular concentration of ROS and by reducing the cellular antioxidant capacity (Pinto et al., 2003).

Differences between estress oxidative biomarkers in control and experimental specimens, at different seasons, have been observed. The higher values of biomarkers such as GST and CAT in autumn with respect to spring season are probably related to the higher ambient temperature, which can lead to an increase in oxygen consumption and therefore to an enhanced ROS generation (Wilhelm Filho et al., 2001). Respect to LPO values, the polyunsaturated fatty acids (PUFA) from membrane phospholipids are the main target of ROS attack, leading to lipid peroxidation (Halliwell and Gutteridge, 1999). It is also known that membranes of cold-acclimated fish are enriched with PUFA, being more susceptible to suffer with an oxidative stress (Parihar et al., 1997). A possible cause of some enzymatic activities increase, such as GP*x* or GR, in spring (lower temperature) is the increased polyunsaturation of mitochondrial membranes in fish. The membranes polyunsaturation can raise mitochondrial respiration rates, which would enhance an ROS formation, increasing proton leak and favoring membranes peroxidation. Higher levels of organic hydroperoxides are formed by an enhanced lipid mobilization, which leads to an induction of higher GP*x* activity. This induces higher utilization of GSH, forming their oxidized form (GSSG) and influences elevated activity of GR, in order to maintain sufficient amount of reduced equivalents in the cells, and thus normal redox homeostasis.

Part of variation recorded for oxidative stress biomarkers cannot be explained by season and could be linked to species, modification of food availability, spawning period, kind of pollution or other environmental factors (Sheehan and Power, 1999).

No correlations between PAHs (sediments and metabolites) with LPO and GR activities were established, but significant negative correlations were established between GST activity and metabolites type naphthalene and pyrene in liver. In the first step of the biotransformation of PAHs, several metabolites are formed, some of which are subject to further transformation by conjugation with endogenous substances. A possible pathway is the conjugation with glutathione, a reaction catalyzed by glutathione-S-transferases (GST), a family of enzymes that is also involved in the lipid peroxidation (LPO) prevention. Glutathione conjugation seems to be an important pathway of detoxification of PAHs, at least in some species, since an induction of GST activity has been found in fish exposed to these xenobiotics. However, an inhibition of GST activity after exposure to PAHs has also been found (Wang et al., 2006; Vieira et al., 2008). Sun et al. (2008) observed in *C. auratus* exposed to 0.001–0.1 mg/L of pyrene, a GST significantly induced at 0.001 and 0.005 mg/L, and then was decreased at higher concentrations. GSH serves as a substrate by conjugation with electrophilic intermediates under the catalytic action of GST. The variation in GSH level can produce variation in GST level. Severe oxidative stress may suppress GSH levels (due to a loss of adaptative mechanisms) and produce the oxidation of GSH to GSSG with a consequent decrease of GST level. Ahmad et al. (2004) observed in *Anguilla anguilla* an increase of GST level after 8 h exposure to harbour water; at 48 h exposure, the naphthalene-type metabolites in liver were lower and the GST activity was similar to activity in the control fish. The GST activity also decreased in gill and kidney, indicating the vulnerability of these organs to the harbour water pollutants.

In sediments, GST was positively correlated with fluoranthene, chrysene and summatory of eight high molecular weight PAHs, in general, HPAHs. Lu et al. (2009) observed in *C. auratus* exposed 15 days to 0.1–10.0 mg/kg fluoranthene, a fold increase of GST activity of 1.46 establishing a clear relationship between GST activity and exposure dosages of PAHs. On the other hand, Pathiratne and Hemachandra (2010) observed an induction of GST only in the lowest dose (1 mg/kg bw) of fluoranthene and chrysene in *Oreochromis niloticus*.

Effects of inducing agents on total hepatic GST activity have been observed in several fish species. As for CYP1A, the mechanism of induction for most GSTs in mammals is regulated via the Ah-receptor (George, 1994). An additional form of GST induction, which functions independently of the Ah-receptor, has been elucidated and requires metabolism of the compound before transcriptional activation of the respective subunit gene can take place (Rushmore and Pickett, 1990).

An increase in hepatic GST activity has been reported in several studies after fish exposure to PAHs (Bello et al., 2001; Ahmad et al., 2004; Jee and Kang, 2005; Jifa et al., 2006; Vieira et al., 2008), but most studies did not demonstrate any significant alterations (Riviere et al., 1990; Lemaire et al., 1996; Fenet et al., 1998; Van Schanke et al., 2002; Sturve et al., 2006; Sun et al., 2006; Oliveira et al., 2008) or showed a decrease of GST activity (Oikari and Jimenez, 1992; Lemaire et al., 1996; Vieira et al., 2008; Sun et al., 2008). Attempts to detect chemically induced activities of GSTs in free living fish also yielded conflicting results. Several studies reported GST activities to be significantly increased (Rodriguez-Ariza et al., 1993a; Vigano et al., 1995; Beyer et al., 1996; Van der Oost et al., 1996, 1998; Machala et al., 2001), but in most cases no significant differences were observed between fish from control and polluted sites (Beyer et al., 1996; Fent et al., 1998; Stanic et al., 2006; Almroth et al., 2008) or a significant decrease in GST activities was observed in some fish species in polluted environments (Van der Oost et al., 1994; Tuvikene et al., 1999).

Hepatic total GST activity in fish does not seem to be feasible as a biomarker for environmental risk assessment, since increased activities are only observed in a limited number of fish species (Van der Oost et al., 2003). In addition, the exposure to pollutants like PAHs may cause both induction and inhibition of the enzyme activity. However, more research on this parameter, which is of paramount importance for major detoxification processes, may elucidate specific isoenzymes that have a more sensitive and selective response to pollutants. Hepatic cytosolic GST activity towards ethacrynic acid appeared to be induced in rainbow trout exposed to PCB 153 or DDE, but was not induced after 2,3,7,8-TCDD exposure (Machala et al., 1998). This observation indicates that this parameter may be a suitable biomarker for exposure to nonplanar PCBs and organochlorines that does not induce the CYP1A activity.

Often, contradictory modulations of potencial biochemical indicators of an oxidative stress in various fish species have been reported (Bainy et al., 1996; Petrivalsky et al., 1997). However, differential induction of the hepatic GST isoenzymes plays an important role in the antioxidant defence. Therefore, more selective GST activities appeared to be additional potential

biomarkers, as shown in both field and short-term laboratory studies (Otto and Moon, 1995; Rodrigez-Ariza et al., 1993b).

GST activities reveal that this biochemical response to pollutant mixtures are complex, since the absence of a specific effect does not always reflect the absence of contamination. Furthermore, the antagonistic effects on liver biotransformation promoted by different xenobiotics present in complex mixtures are well known. Finally, the role of GST on PAHs detoxification in fish deserves further research.

GP*x* showed a negative significant correlation with metabolites type phenanthrene in liver and positive correlation with acenaphthene concentration in sediments.

Normally, laboratory and field studies have shown increases in the GP*x* activity after exposure of fish to different pollutants (Van der Oost et al., 2003). Yin et al. (2007) identified the reactive oxygen specie generated in *C. auratus* by phenanthrene as hydroxyl radical (· OH). Jee and Kang (2005) reported a remarkable increase in the activity of hepatic GP*x* in *Paralichthys olivaceus* exposed to 2.0 μM phenanthrene after 2 weeks exposure. Oliveira et al. (2008) observed in *Liza aurata* exposed to 0.3, 0.9 and 2.7 μM phenanthrene for a period of 16 h a significant increase of GP*x* activity. Recently, López-Galindo et al. (2010a) observed an increase of GP*x* activity in *S. Senegalensis* exposed to 2 mg/L Mexel®432 (a mixture of aliphatic hydrocarbons with an alcohol and amine functionality). However, the same authors (López-Galindo et al., 2010b) did not observe any trend of the GP*x* activity over time and dose in *S. senegalensis* exposed to 0.1 mg/L hypochlorite. A significant decrease in GP*x* activity was observed in field experiments with red mullet, *Mullus barbatus* (Burgeot et al., 1996) and seabream *Diplodus annularis* (Bagnasco et al., 1991) exposed to PAHs contaminated sites.

Glutathione peroxidase is the most important peroxidase for the detoxification of hydroperoxides and of hydrogen peroxide. The GP*x* enzyme has been postulated to protect erythrocytes from damage by H_2O_2 and responsible for reduction of lipid hydroperoxides. Therefore, it is hypothesized that this enzyme may protect tissues against an oxidative damage due to lipid peroxidation. The liver is a major site of detoxification and the first target of ingested oxidants, and is considered to be a very important tissue in the study of GP*x* protective role from lipid peroxidation.

It is not a common negative correlation between GP*x* activity and pollutants. To explain the negative correlation between GP*x* activity and phenanthrene-type metabolites in liver, results observed by authors have been compared with the effect of phenanthrene (concentrations in mg/mL water) on the fish GP*x* activity in laboratory bioassays, but there is no sufficient bibliography available about the relation between GP*x* and phenanthrene-type metabolites in liver from field assays. One explanation could be a suppression of the GP*x* inducibility, due to chronic exposure to complex mixture of pollutants with the presence of GP*x* inhibitors compounds, such us mercury, cadmium or copper (Ulusu et al., 2002; Ahmad et al., 2005; Vicente-Martorell et al., 2009; Franco et al., 2009); other explanation could be that, since GST is a cofactor for glutathione peroxidase, the decrease of GST (that present a negative correlation with naphthalene-type metabolites in liver) to face oxidative stress produce a decrease of Gp*x* activity (Vieira et al., 2009). More research is required to determine the potential utility of GP*x* activity in fish liver as a biomarker for environmental risk assessment purposes.

A significant correlation of CAT with anthracene concentration in sediment was observed. CATs are hematin-containing enzymes that facilitate the removal of hydrogen peroxide (H_2O_2), which is metabolized to molecular oxygen (O_2) and water. Since CATs are localized in the peroxisomes of most cells and are involved in fatty acid metabolism, changes in activities may often be difficult to interpret (Stegeman et al., 1992). Sturve et al. (2006) also found a significant increase of CAT activity in the Atlantic cod (*G. morhua*) exposed to North Sea oil. Vieira et al. 2008 observed a significant difference between the control and fish (*P. microps*) exposed to 2 μg/L anthracene with a percentage of induction of 58%. These results suggest that anthracene induce the production of O_2^-, which is converted to hydrogen peroxide (H_2O_2) by an action of CAT.

The mechanisms of toxicity and detoxification of PAHs in fish are not fully understood and even contradictory effects of PAHs on antioxidant enzymes have been reported depending on the substance, time exposure and concentration. (Vieira et al., 2008). Therefore, since antioxidant enzymes of fish have been used as biomarkers in areas polluted with petrochemical products, it is convenient to clarify their pattern of answer to petrochemical products and their components.

5. Conclusions

Using the principal reference in Europe on sediment quality (Holland Legislation) Odiel and Tinto River are no or few contaminated sites and Padre Santo Canal is a site moderately contaminated PAHs site. However, higher responses in the activities of the biomarkers were observed in Tinto and Odiel River, probably due to the presence of other pollutants, such as heavy metals and/or pesticides.

LPO, CAT and GR biomarkers were most sensitive to chronic pollution of complex mixtures showing a significant difference with respect to the control fish in Odiel and Tinto Rivers. Nevertheless, GST, CAT and GP*x* showed correlations with both, sediments and liver PAHs.

GST was most correlated with four rings PAHs and both, GP*x* and CAT, was most correlated with three rings PAHs.

The field studies are important because isolated PAHs may be inducers of anti-oxidant enzymes, but complex mixtures can contain compounds that affect (direct and/or indirect) to the enzyme activities producing different effects with respect to laboratory assays realized with the same compounds and enzymes.

Acknowledgments

This work has been supported by Project SEAPAT: "Biomarkers and histopathology in gilthead seabream, *Sparus aurata* and Senegal sole, *Solea senegalensis*" (CICYT, REN 2003-09361-CO3-03).

References

Adams, S.M., Shepard, K.L., Greely Jr, M.S., Jimenez, B.D., Ryon, M.G., Shugart, L.R., McCarthy, F.F., Hinton, D.E., 1989. The use of bioindicators for assessing the effects of pollutant stress on fish. Mar. Environ. Res. 28, 459–464.

Ahmad, I., Pacheco, M., Santos, M.A., 2004. Enzymatic and nonenzymatic antioxidants as an adaptation to phagocyte-induced damage in *Anguilla anguilla* L. following in situ harbour water exposure. Ecotoxicol. Environ. Saf. 57, 290–302.

Ahmad, I., Oliveira, M., Pacheco, M., Santos, M.A., 2005. *Anguilla anguilla* L. oxidative stress biomarkers responses to copper exposure with or without β-naphthoflavone pre-exposure. Chemosphere 61 (2), 267–275.

Almroth, B.C., Sturve, J., Stephensen, E., Holth, T.F., Förlin, L., 2008. Protein carbonyls antioxidant defenses in corkwing wrasse (*Symphodus melops*) from a heavy metal polluted and a PAH polluted site. Mar. Environ. Res. 66, 272–277.

Bagnasco, M., Camoirano, A., De Flora, S., Melodia, F., Arillo, A., 1991. Enhanced liver metabolism of mutagens and carcinogens in fish living in polluted seawater. Mutat. Res. 262, 129–137.

Bainy, A.C.D., Saito, E., Carvalho, P.S.M., Junqueira, V.B.C., 1996. Oxidative stress in gill, erythrocytes, liver and kidney of Nile tilapia (*Oreochromis niloticus*) from a polluted site. Aquat. Toxicol. 34, 151–162.

Bello, S.M., Franks, D.G., Stegeman, J.J., Hahn, M.E., 2001. Acquired resistence to Ah receptor agonists in a population of Atlantic killfish (*Fundulus heteroclitus*)

inhabiting a marine superfund site: in vivo and in vitro studies on the inducibility of xenobiotic metabolizing enzymes. Toxicol. Sci. 60, 77–91.
Beyer, J., Sandvik, M., Hylland, K., Fjeld, E., Egaas, E., Aas, E., Skaare, J.U., Goksoir, A., 1996. Contaminant accumulation and biomarker responses in flounder (*Platichthys flesus* L.) and Atlantic cod (*Gadus morhua* L.) exposed by caging to polluted sediments in Sorfjorden, Norway. Aquat. Toxicol. 36, 75–98.
Bird, R.P., Draper, A.H., 1984. Comparative studies on different methods of malondyhaldehyde determination. Method Enzymol. 90, 105–110.
Burton, J.G.A., 2002. Sediment quality criteria in use around the world. Limnology 3, 65–76.
Bradford, M.M., 1976. A rapid and sensitive method for quantification of microgram quantities of protein utilizing the principle of protein-dye-binding. Anal. Biochem. 72, 248–254.
Burgeot, T., Bocquené, G., Porte, C., Dimeet, J., Santella, R.M., Garcia de la Parra, L.M., Pftol-Leszkowics, A., Raoux, C., Galgani, F., 1996. Bioindicators of pollutant exposure in the northwestern Mediterranean Sea. Mar. Ecol. Prog. Ser. 131, 125–141.
Carlson, E.A., Li, Y., Zelikoff, J.T., 2004. Benzo(a)pyrene-induced immunotoxicity in Japanese medaka (*Oryzias latipes*): relationship between lymphoid CYP1A activity and humoral immune suppression. Toxicol. Appl. Pharmacol. 201 (1), 40–52.
Cavalieri, E., Rogan, E., 1985. Role of radical cations in aromatic hydrocarbons carcinogenesis. Environ. Health Perspect. 64, 69–84.
Claiborne, A., 1985. Catalase activity. In: Greenwald, R.A. (Ed.), CRC Handbook of Methods in Oxygen Radical Research. CRC Press, Boca Raton, Fl, pp. 283–284.
Conell, D.W., Miller, G.J., 1984. Chemistry and Ecotoxicology of Pollution. Willey-Interscience Publishers, USA 444 pp.
Cossu, C., Doyotte, A., Jacquin, M.C., Babut, M., Exinger, A., Vasseur, P., 1997. Glutathione reductase, selenium-dependent glutathione peroxidase, glutathione levels, and lipid peroxidation in freshwater bivalves, *Unio tumidus*, as biomarkers of aquatic contamination in field studies. Ecotoxicol. Environ. Saf. 38, 122–131.
Costa, P.M., Diniz, M.S., Caeiro, S., Lobo, J., Martins, M., Ferreira, A.M., Caetano, M., Vale, C., Del Valls, T.A., Costa, M.H., 2009. Histological biomarkers in liver and gills of juvenile *Solea senegalensis* exposed to contaminated estuarine sediments: a weighted indices approach. Aquat. Toxicol. 92 (3), 202–212.
Cribb, A.E., Leeder, J.S., Spielberg, S.P., 1989. Use of a microplate reader in assay of glutathione reductase using 5,5′-dithiobis(2-nitrobenzoic acid). Anal. Biochem. 183, 195–196.
Dautremepuits, C., Betoulle, S., Vernet, G., 2002. Antioxidant response modulated by copper in healthy or parasitized carp (*Cyprinus carpio* L.) by Ptychobothrium sp. (Cestoda). BBA-GEN Subj. 1573, 4–8.
Den Besten, P.J., Heise, S., 2007. Risk assessment approaches in European countries. Sustainable Manage. Sediment Resour. 3, 153–205.
Dinis, M.T., 1992. Aspects of the potential of *Solea senegalensis* Kaup for aquaculture: larval rearing and weaning to an artificial diet. Aquacult. Fish. Manag. 23, 179–183.
Di Giulio, R.T., Habig, C., Gallager, E.P., 1993. Effects of Black Rock Harbor sediments on indices of biotransformation, oxidative stress, and DNA integrity in channel catfish. Aquat. Toxicol. 26, 1–22.
Drake, P., Arias, A.M., Rodríguez., A., 1984. Cultivo extensivo de peces marinos en los esteros de las salinas de San Fernando (Cádiz): II. Características de la producción de peces. Fish. Res. Inst. (Spain) (Tech. Rep.) 116, 1–23.
Eisler, R., 1987. Polycyclic aromatic hydrocarbon hazards to fish, wildlife and invertebrates. A synoptic review: Laural, Md., Patuxent Wildlife Research Centre. U.S. Fish and Wildlife Service. Biological Report. 85 (1.11), 81.
Fenet, H., Casellas, C., Bontoux, J., 1998. Laboratory and field-caging studies on hepatic enzymatic activities in European eel and rainbow trout. Ecotoxicol. Environ. Saf. 40, 137–143.
Fent, K., Woodin, B.R., Stegeman, J.J., 1998. Effects of triphenyltin and other organotins on hepatic monooxygenase system in fish. Comp. Biochem. Physiol. C Pharmacol. Toxicol. Endocrinol. 121, 277–288.
Filho, D.W., Tribess, T., Gáspari, C., Cláudio, F.D., Torres, M.A., Magalhaes, A.R.M., 2001. Seasonal changes antioxidant defences of the digestive gland of the brown mussel (*Perna perna*). Aquaculture 203, 149–158.
Franco, J.L., Posser, T., Dunkley, P.R., Dickson, P.W., Mattos, J.J., Martins, R., Bainy, A.C., Marques, M.R., Dafre, A.L., Farina, M., 2009. Methylmercury neurotoxicity is associated with inhibition of the antioxidant enzyme glutathione peroxidase. Free Radical Biol. Med. 47 (4), 449–457.
Froese R., Pauly, D., 2010. Fishbase. Worl Wide Web Electronic Publication. 〈www.fishbase.org〉.
Gagnon, M.M., Holdway, D.A., 1999. Metabolic enzyme activities in fish gills as biomarkers of exposure to petroleum hydrocarbons. Ecotoxicol. Environ. Saf. 44, 92–99.
Gagnon, M.M., Holdway, D.A., 2000. EROD induction and biliary metabolite excretion following exposure to the water accommodated fraction of crude oil and to chemically dispersed crude oil. Arch. Environ. Contamination Toxicol. 38 (1), 70–77.
George, S.G., 1994. Enzymology and molecular biology of phase II xenobiotic-conjugating enzymes in fish. In: Malins, D.C., Ostrander, G.K. (Eds.), Aquatic Toxicology; Molecular, Biochemical and Cellular Perspectives. Lewis Publishers, CRC Press, pp. 37–85.
Gravato, C., Santos, M.A., 2003. *Dicentrarchus labrax* biotransformation and genotoxicity responses after exposure to a secondary treated industrial/urban effluent. Ecotoxicol. Environ. Saf. 55, 300–306.
Habig, W.H., Pabst, M.J., Jakoby, W.B., 1974. Glutathione-S-transferases, the first enzymatic step in mercapturic acid formation. J. Biol. Chem. 249, 7130–7139.
Halliwell, B., Gutteridge, J.M.C., 1999. Free Radicals in Biology and Medicine. Oxford University Press, Oxford.
Jee, J.H., Kang, J.C., 2005. Biochemical changes in enzymatic defense system after phenanthrene exposure in olive flounder, *Paralichthys olivaceus*. Physiol. Res. 54, 585–591.
Jifa, W., Zhimin, Y., Xiuxian, S., You, W., 2006. Response of integrated biomarkers of fish (*Lateolabrax japonicus*) exposed to benzo(a)pyrene and sodium dodecylbenzene sulfonate. Ecotoxicol. Environ. Saf. 65, 230–236.
Jiménez-Tenorio, N., Morales-Caselles, C., Kalman, J., Salamanca, M.J., González de Canales, M.L., Sarasquete, C., Del Valls, T.A., 2007. Determining sediment quality for regulatory proposes using fish chronic bioassays. Environ. Int. 33, 474–480.
Jimenez-Tenorio, N., Salamanca, M.J., Garcia-Luque, E., Gonzalez de Canales, M.L., Del Valls, T.A., 2008. Chronic bioassay in benthic fish for the assessment of the quality of sediments in different areas of the coast of Spain impacted by acute and chronic oil spills. Environ. Toxicol. 23 (5), 634–642.
Kamer, T.T., 1994. Dutch standards for contaminated sediments. Evaluatienota Water 21 (250), 27–28.
Klaunig, J.E., Xu, Y., Isenberg, J.S., Bachowski, S., Kolaja, K.L., Jiang, A., Stevenson, D.E., Walborg, E.F., 1998. The role of oxidative stress in chemical carcinogenesis. Environ. Health Perspect. 106, 289–295.
Landis, G., Yu, M.H., 1995. Introduction to Environmental Toxicology: Impacts of Chemical Upon Ecological Systems. Lewis, Boca Raton, FL.
Lemaire, P., Förlin, L., Livingstone, D., 1996. Responses of hepatic biotransformation and antioxidant enzymes to CYP1A-inducers (3-methylcholanthrene, β-naphthoflavone) in sea bass (*Dicentrarchus labrax*), dab (*Limanda limanda*) and rainbow trout (*Oncorhynchus mykiss*). Aquat. Toxicol. 36, 141–160.
López-Galindo, C., Vargas-Chacoff, L., Nebot, E., Casanueva, J.F., Rubio, D., Solé, M., Mancera, J.M., 2010a. Sublethal effects of the organic antifoulant Mexel®432 on osmsoregulation and xenobiotic detoxification in the flatfish *Solea senegalensis*. Chemosphere 79, 78–85.
López-Galindo, C., Vargas-Chacoff, L., Nebot, E., Casanueva, J.F., Rubio, D., Solé, M., Mancera, J.M., 2010b. Biomarker responses in *Solea senegalensis* exposed to sodium hypochlorite used as antifouling. Chemosphere 78, 885–893.
Lu, G.H., Wang, C., Zhu, Z., 2009. The dose–response relationships for EROD and GST induced by polyaromatic hydrocarbons in *Carassius auratus* B. Environ. Contamination Toxicol. 82, 194–199.
Machala, M., Drabeck, P., Neca, J., Kolarova, J., Svobodova, Z., 1998. Biochemical markers for differentiation of exposures to nonplanar polychlorinated biphenyls, organochlorine pesticides, or 2,3,7,8-tetrachlorodibenzo-p-dioxin in trout liver. Ecotoxicol. Environ. Saf. 41, 107–111.
Machala, M., Dusek, L., Hilscherova, L., Kubinova, R., Jurajda, P., Neca, J., Ulrich, R., Gelnar, M., Studnickova, Z., Holoubek, I., 2001. Determination and multivariate statistical analysis of biochemical responses to environmental contaminants in feral freshwater fish *Leuciscus cephalus* L. Environ. Toxicol. Chem. 20 (5), 1141–1148.
Mohandas, J., Marshall, J.J., Duggins, G.G., Horvath, J.S., Tiller, D., 1984. Differential distribution of glutathione and glutathione related enzymes in rabbit kidney. Possible implications in analgesic neuropathy. Cancer Res. 44, 5086–5091.
Narbonne, J.F., Djomo, J.E., Ribera, D., Ferrier, V., Garrigues, P., 1999. Accumulation kinetics of polycyclic aromatic hydrocarbons adsorbed to sediment by the mollusk *Corbicula fluminea*. Ecotoxicol. Environ. Saf. 42, 1–8.
Ohkawa, H., Ohisi, N., Yagi, K., 1979. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Anal. Biochem. 95, 351–358.
Oikari, A., Jimenez, B., 1992. Effects of hepatotoxicants on the induction of microsomal monooxygenase activity in sunfish liver by β-naphtoflavone and benzo(a)pyrene. Ecotoxicol. Environ. Saf. 23, 89–102.
Oliva, M., Garrido, M.C., Sales Márquez, D., González de Canales, M.L., 2009. Sublethal and lethal toxicity in juvenile Senegal sole (*Solea senegalensis*) exposed to copper: a preliminary toxicity range-finding test. Exp. Toxicol. Pathol. 61 (2), 113–121.
Oliveira, M., Pacheco, M., Santos, M.A., 2008. Organ specific antioxidant responses in golden grey mullet (*Liza aurata*) following a short-term exposure to phenanthrene. Sci. Total Environ. 396, 70–78.
Otto, D.M.E., Moon, T.W., 1995. 3,3',4,4'-Tetrachlorobiphenyl effects on antioxidant enzymes and glutathione status in different tissues of rainbow trout. Pharmacol. Toxicol. 77, 281–287.
Parihar, M.S., Javeri, T., Hemnani, T., Dubey, A.K., Prakash, P., 1997. Responses of superoxide dismutase, glutathione peroxidase and glutathione antioxidant defenses in gills of the freshewater catfish (*Heteropneustes fossilis*) to short-term elevated temperature. J. Therm. Biol. 22, 151–156.
Pathiratne, A., Hemachandra, C.K., 2010. Modulation of ethoxyresorufin O-deethylase and glutathione s-transferase activities in Nile tilapia (*Oreochromis niloticus*) by polycyclic aromatic hydrocarbons containing two to four rings: implications in biomonitoring aquatic pollution. Ecotoxicology. Published online 13 March.
Petrivalsky, M., Machala, M., Nezveda, K., Piacka, V., Svobodova, z., Drabek, P., 1997. Glutathione dependent detoxifying enzymes in rainbow trout liver: search for specific biochemical markers of chemical stress. Environ. Toxicol. Chem. 16, 1417–1421.
Pinto, E., Sigaud-Kutner, T.C.S., Leitão, M.A.S., Okamoto, O.K., Morse, D., Colepicolo, P., 2003. Heavy metal-induced oxidative stress in algae. J. Phycol. 39, 1008–1018.

Reynaud, S., Marionnet, D., Taysse, L., Duchiron, C., Deschaux, P., 2002. The effects of 3-methylcholanthrene on macrophage respiratory burst and biotransformation activities in the common carp (*Cyprinus carpio* L.). Fish Shellfish Immunol. 12 (1), 17–34.

Reynaud, S., Raveton, M., Ravanel, P., 2008. Interactions between immune and biotransformation systems in fish: a review. Aquat. Toxicol. 87, 139–145.

Riviere, J.L., Devaux, A., Goñi, O., Monod, G., 1990. Effect of β-naphtoflavone and MCPA on liver and kidney drug-metabolizing enzymes from the carp, *Cyprinus carpio*. Ecotoxicol. Environ. Saf. 19, 276–284.

Roche, H., Bogé, G., 1993. *In vitro* effects of Cu, Zn and Cr salts on antioxidant enzyme activities of red blood cells of a marine fish *Dicentrarchus labrax*. Toxicology 7, 623–629.

Rodriguez-Ariza, A., Martinez-Lara, E., Pascual, P., Pedradas, J.R., Abril, N., Dorado, G., Toribio, F., Barcena, J.A., Peinado, J., Pueyo, C., Lopez-Barea, J., 1993a. Biochemical and genetic indices of marine pollution in Spanish littoral. Sci. Total Environ. 134 (Suppl. 1), 109–116.

Rodrigez-Ariza, A., Peinado, J., Pueyo, C., Lopez Barea, J., 1993b. Biochemical indicators of oxidative stress in fish from polluted littoral areas. Can. J. Fish Aquat. Sci. 50, 2568–2573.

Rushmore, T.H., Pickett, C.B., 1990. Transcriptional regulation of the rat gluthatione S-transferase Ya subunit gene. J. Biol. Chem. 265, 1250–1257.

Salazar-Coria, L., Amezcua-Allieri, M.A., Tenorio-Torres, M., González-Macías, C., 2007. Polyaromatic hydrocarbons (PAHs) and metal evaluation after a diesel spill in Oaxaca, Mexico. Bull. Environ. Contamination Toxicol. 79, 462–467.

Sarkar, A., Ray, D., Shrivastava, A.N., Sarker, S., 2006. Molecular biomarkers: their significance and application in marine pollution monitoring. Ecotoxicology 15, 333–340.

Schrap, S.M., Opperhuizen, A., 1990. Relationship between bioavailability and hydrophobicity: reduction of the uptake of organic chemicals by fish due to the sorption on particles. Environ. Toxicol. Chem. 9, 715–724.

Sheehan, D., Power, A., 1999. Effects of seasonality on xenobiotic and antioxidant defense mechanisms of bivalve molluscs. Comp. Biochem. Physiol. C 123, 193–199.

Shi, H., Sui, Y., Wang, X., Luo, Y., Liangliang, J., 2005. Hydroxyl radical production and oxidative damage induced by cadmium and naphthalene in liver of *Carassius auratus*. Comp. Biochem. Physiol. C. 140, 115–121.

Silva, C.A., Oliveira, C.A., Katsumiti, A., Araujo, M.L.P., Zandoná, E.M., Costa, G.P., Maschio, J., Roche, H., Silva de Assis, H.C., 2009. Evaluation of waterborne exposure to oil spill 5 years after an accident in Southern Brazil. Ecotoxicol. Environ. Saf. 72 (2), 400–409.

Solé, M., Lima, D., Reis-Henriques, M.A., Santos, M.M., 2008. Stress biomarkers in juvenile Senegal sole, *Solea senegalensis*, exposed to the water-accommodated fractions of the "Prestige" fuel oil B. Environ. Contamination Toxicol.

Stanic, B., Andric, N., Zoric, S., Grubor-Lajsic, G., Kovacevik, R., 2006. Assessing pollution in the Danube river near Novi Sad (Serbia) using several biomarkers in starlet (*Acipenser ruthenus* L.). Ecotoxicol. Environ. Saf. 65, 395–402.

Stegeman, J.J., Brouwer, M., Richard, T.D.G., Förlin, L., Fowler, B.A., Sanders, B.M., Van Veld, P.A., 1992. Molecular responses to environmental contamination: enzyme and protein systems as indicators of chemical exposure and effect. In: Huggett, R.J., Kimerly, R.A., Mehrle Jr., P.M., Bergman, H.L. (Eds.), Biomarkers: Biochemical, Physiological and Histological Markers of Anthropogenic Stress. Lewis Publishers, Chelsea, MI, USA, pp. 235_/335.

Stein, J.E., Collier, T.K., Reicert, W.L., Casillas, E., Hom, T., Varanasi, U., 1992. Bioindicators of contaminant exposure and sublethal effects: studies with benthic fish in Puget Sound, Washington. Environ. Toxicol. Chem. 11, 701–714.

Sturve, J., Hasselberg, L., Fälth, H., Celander, M., Förlin, L., 2006. Effects of North Sea oil and alkylphenols on biomarker responses in juvenile Atlantic cod (*Gadus morhua*). Aquat. Toxicol. 78S, S73–S78.

Sun, Y., Yu, H., Zhang, J., Yin, Y., Shi, H., Wang, X., 2006. Bioaccumulation, depuration and oxidative stress in fish *Carassius auratus* under phenanthrene exposure. Chemosphere 63, 1319–1327.

Sun, Y., Yin, Y., Zhang, J., Yu, H., Wang, X., Wu, J., Xue, Y., 2008. Hydroxyl radical generation and oxidative stress in *Carassius auratus* liver, exposed to pyrene. Ecotoxicol. Environ. Saf. 71, 446–453.

Torres, M.A., Testa, C.P., Gáspari, C., Masutti, M.B., Panitz, C.M.N., Curi-Pedrosa, R., Almeida, E.A., Di Mascio, P., Filho, D.W., 2002. Oxidative stress in the mussel *Mytella guyanensis* from polluted mangroves on Santa Catarina Island, Brazil. Mar. Pollut. Bull. 44, 923–932.

Tuvikene, A., 1995. Responses of fish to polycyclic aromatic hydrocarbons (PAHs). Ann. Zool. Fennici 32, 295–309.

Tuvikene, A., Huuskonen, S., Koponen, K., Ritola, O., Mauer, U., Lindstrom-Seppa, P., 1999. Oil shale processing as a course of aquatic pollution: monitoring of the biologic effects in caged and feral freshwater fish. Environ. Health Perspect. 107, 745–752.

Ulusu, N., ACAN, L., Turan, B., Tezcan, E.F., 2002. Inhibition of glutathione reductase by cadmium ion in some rabbit tissues and the protective role of dietary selenium. Biol. Trace Elem. Res. 91 (2), 151–156.

USEPA, 1996. United States Environmental Protection Agency. Method 3540C: Soxhlet extraction. 〈www.epa.gov〉.

USEPA, 2000. United States Environmental Protection Agency. Method 3620C: Florisil cleanup. 〈www.epa.gov〉.

Van der Oost, R., Van Schooten, F.J., Ariese, F., Heida, H., Vermeulen, N.P.E., 1994. Bioaccumulation, biotransformation and DNA binding of PAHs in feral eel (*Anguilla anguilla*) exposed to polluted sediments: a field survey. Environ. Toxicol. Chem. 13, 859–870.

Van der Oost, R., Goksoyr, A., Celander, M., Heida, H., Vermeulen, N.P.E., 1996. Biomonitoring aquatic pollution with feral eel (*Anguilla anguilla*): II. Biomarkers: pollution-induced biochemical responses. Aquat. Toxicol. 36, 189–222.

Van der Oost, R., Lopes, S.C.C., Komen, H., Satumalay, K., Van den Bos, R., Heida, H., Vermeulen, N.P.E., 1998. Assessment of environmental quality and inland water pollution using biomarker responses in caged carp (*Cyprinus carpio*); use of a bioactivation-detoxification ratio as biotransformation index (BFI). Mar. Environ. Pollut. 46, 315–319.

Van der Oost, R., Beyer, J., Vermeulen, N.P.E., 2003. Fish bioaccumulation and biomarkers in environmental risk assessment: a review. Environ. Toxicol. Pharmacol. 13, 57–149.

Van Schanke, A., Boon, J.P., Aardoom, Y., Van Leest, A., Van Schooten, F.J., Maas, L., Van den Berg, M., Everaarts, J., 2002. Effect of dioxin-like PCB (CB 126) on the biotransformation and genotoxicity of benzo(a)pyrene in the marine flatfish (*Limanda limanda*). Aquat. Toxicol. 50, 403–415.

Varanasi, U., Stein, J.E., 1991. Disposition of xenobiotic chemicals and metabolites in marine organisms. Environ. Health Perspect. 90, 93–100.

Vicente-Martorell, J.J., Galindo-Riaño, M.D., García-Vargas, M., Castro-Granado, D., 2009. Bioavailability of heavy metals monitoring water, sediments and fish species from a polluted estuary. J. Hazard Mater. 162, 823–836.

Vieira, L.R., Sousa, A., Frasco, M.F., Lima, I., Morgado, F., Guilhermino, L., 2008. Acute effects of Benzo(a)pyrene, anthracene and a fuel oil on biomarkers of the common goby Pomatoschistus microps. Sci. Total Environ. 395, 87–100.

Vieira, L.R., Gravato, C., Soares, A.M.V.M., Morgado, F., Guilhermino, L., 2009. Acute effects of copper and mercury on the estuarine fish *Pomatoschistus microps*: linking biomarkers to behaviour. Chemosphere 76 (10), 1416–1427.

Vigano, L., Arillo, A., Melodia, F., Bagnasco, M., Bennicelli, C., De Flora, S., 1995. Hepatic and biliary biomarkers in rainbow trout injected with sediment extracts from the river Po (Italy). Chemosphere 30, 2117–2128.

VROM, 2000. Netherlands Ministry of Housing Spatial Planning and the Environment. Circular on target values and intervention values for soil remediation. 〈http://international.vrom.nl/Docs/international/annexS_I2000.pdf〉.

Wang, C., Zhao, Y., Zheng, R., Ding, X., Wei, W., Zuo, Z., et al., 2006. Effects of tributyltin, benzo(a)pyrene, and their mixture on antioxidant defence systems in Seabastiscus marmoratus. Ecotoxicol. Environ. Saf. 65, 381–387.

Wilhelm Filho, D., Torres, M.A., Tribess, T.B., Pedrosa, R.C., Soares, C.H., 2001. Influence of season and pollution on the antioxidant defenses of the cichlid fish (*Geophagus brasiliensis*). Brazilian J. Med. Biol. Res. 34 (6), 19–26.

Yin, Y., Jia, H., Sun, Y., Yu, H., Wang, X., Wu, J., Xue, Y., 2007. Bioaccumulation and ROS generation in liver of *Carassius auratus*, exposed to phenanthrene. Comp. Biochem. Physiol. C. 145, 288–293.